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Award Number: W81XWH-06-1-0598

TITLE: The Role Of Alternative Splicing In Breast Cancer Progression

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REPORT DATE: September 2007

TYPE OF REPORT: Revised Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-09-2007	Revised Final	1 SEP 2006 - 31 AUG 2007

4. TITLE AND SUBTITLE

The Role Of Alternative Splicing In Breast Cancer Progression

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0598

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of California, Irvine
Irvine, CA 92697-4024

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Alternative pre-mRNA splicing generates thousands of different mRNA isoforms in metazoan organisms. It is unknown if breast-cancer-associated alternative splicing is regulated like tissue-specific splicing, or whether it is caused by changes in the splicing accuracy. To test the hypothesis that the accuracy of the spliceosome is compromised in breast tumor cells, we have designed a quantitative real-time PCR assay to determine the number of incorrectly spliced mRNA products made from pre-mRNA transcripts that produce only a single mRNA product in all eukaryotic genomes. Analysis of all possible alternative exon exclusion patterns for these genes demonstrates that in some cases a splicing mistake is made only once in 25,000 intron removal events. These results demonstrate that the error rate of the spliceosome is extremely low. Using this assay we examined splicing error rates in breast cancer cell lines. Using matched cancer and normal cell lines we demonstrated that breast cancer cell lines exhibit an up to 3-fold decrease in the number of splicing errors. We conclude that perturbed pre-mRNA splicing in breast cancer is mediated in part through alterations of the intrinsic fidelity of the spliceosome.

15. SUBJECT TERMS
Alternative splicing, breast cancer, spliceosome fidelity

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	9	**19b. TELEPHONE NUMBER** *(include area code)*

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Introduction:

Changes in alternative pre-mRNA splicing are associated with multiple types of cancer, including breast cancer. However, two important questions remain to be answered. First, do these changes in splicing actually contribute to cancer? Simplistically, are they 'causes' or merely 'symptoms' of tumorigenesis? At least some cancer-specific splicing variants appear to make important functional contributions to the transformed state, such as inhibiting apoptosis or blocking tumor suppressor activity. The second question concerns the character of the splicing change itself. Does the alternative splicing pattern derive from a regulated switch, like tissue-specific splicing, or does it result from a loss in splicing accuracy? For example, breast cancers show a large increase in alternatively splicing, whereas in normal cells such failures occur infrequently. During the period of support we addressed the second important question and tested the hypothesis that the accuracy of the spliceosome is compromised in breast tumor cells. The results from our studies demonstrated that breast cancer induces changes in the ability of the splicing machinery to faithfully remove introns.

Body:

The research accomplishments will be described according to the tasks outlined in the original proposal:

Task 1. Determine the error frequency of the splicing machinery through analysis of *Uba52* pre-mRNA splicing.

We have completed this task and determined the error frequency of Uba52, a ribosomal associated gene that based on several stringent criteria is constitutively spliced. To do so, we prepared high quality cDNA from HeLa cells grown to confluency, designed and tested a series of exon junction primers specific for amplification of correct and erroneous splicing events, and carried out a sensitive real-time PCR assays to determine the relative frequency of all possible *Uba52* mRNA isoforms. This analysis demonstrated that at best only 1 in 25,000 intron removal events results in an erroneous mRNA isoform. We concluded from these experiments that the spliceosome carries out intron removal with high fidelity (Figure 1).

Task 2. Determine the alternative splicing frequency for, a gene associated with the regulation of apoptosis.

The completion of the Uba52 analysis demonstrated that the spliceosome may be capable of carrying out intron removal with a high level of accuracy. To ensure that such an interpretation is valid, we felt it was necessary to carry out a splicing error analysis using an additional gene that meets the stringent criteria of being constitutively spliced. We acknowledge that our initial proposal was to analyze alternative splicing of *survivin*, a gene associated with the regulation of apoptosis. However, exhaustive EST database analysis indicated that *survivin* is involved in frequent and complex alternative splicing. Thus, while an analysis of *survivin* splicing would have resulted in obtaining additional information about the frequency of its already established alternative splicing patterns, insufficient data points would have been collected to verify the intrinsic level of spliceosomal accuracy. Instead of *survivin*, we analyzed another highly conserved and constitutively spliced gene, *RPL23*. After designing all possible exon/exon junction primers for *RPL23*, we determined the specificity of amplification for all primer pairs. High quality cDNA from HeLa cells grown to confluency was then generated to carry out real-time PCR assays to determine the relative frequency of all possible *RPL23* mRNA isoforms. Interestingly, correlating the accuracy of *RPL23* pre-mRNA splicing with accuracy of *Uba52* pre-mRNA splicing indicated that the same maximal splicing accuracy, i.e. 1 error in approximately 25,000 splicing events, was observed (Figure 1). Further analyses controlling for the effects of differential mRNA export and degradation suggested that the splicing accuracy is actually limited by the accuracy of transcription by Pol II. That is, transcription mistakes at splice sites occur ~1 in 20,000 times, thus resulting in an exonic region within a pre-mRNA that cannot be recognized by the spliceosome. We conclude from Tasks 1&2 that the accuracy of pre-mRNA splicing is limited by Pol II transcription. Alternative splicing of *survivin* will be investigated in the future using microarray analysis (see below).

Three important conclusions can be drawn from these results. First, the spliceosome recognizes and removes introns with an astonishingly high degree of accuracy that is limited by the quality of pre-mRNAs generated by RNA pol II. Second, alternative splicing is a feature of all multi-intron pre-mRNAs resulting in mRNA isoforms that represent all possible exon ligation combinations. Third, the high levels of alternative splicing observed in the human genome are the consequence of sub-optimal splicing signals.

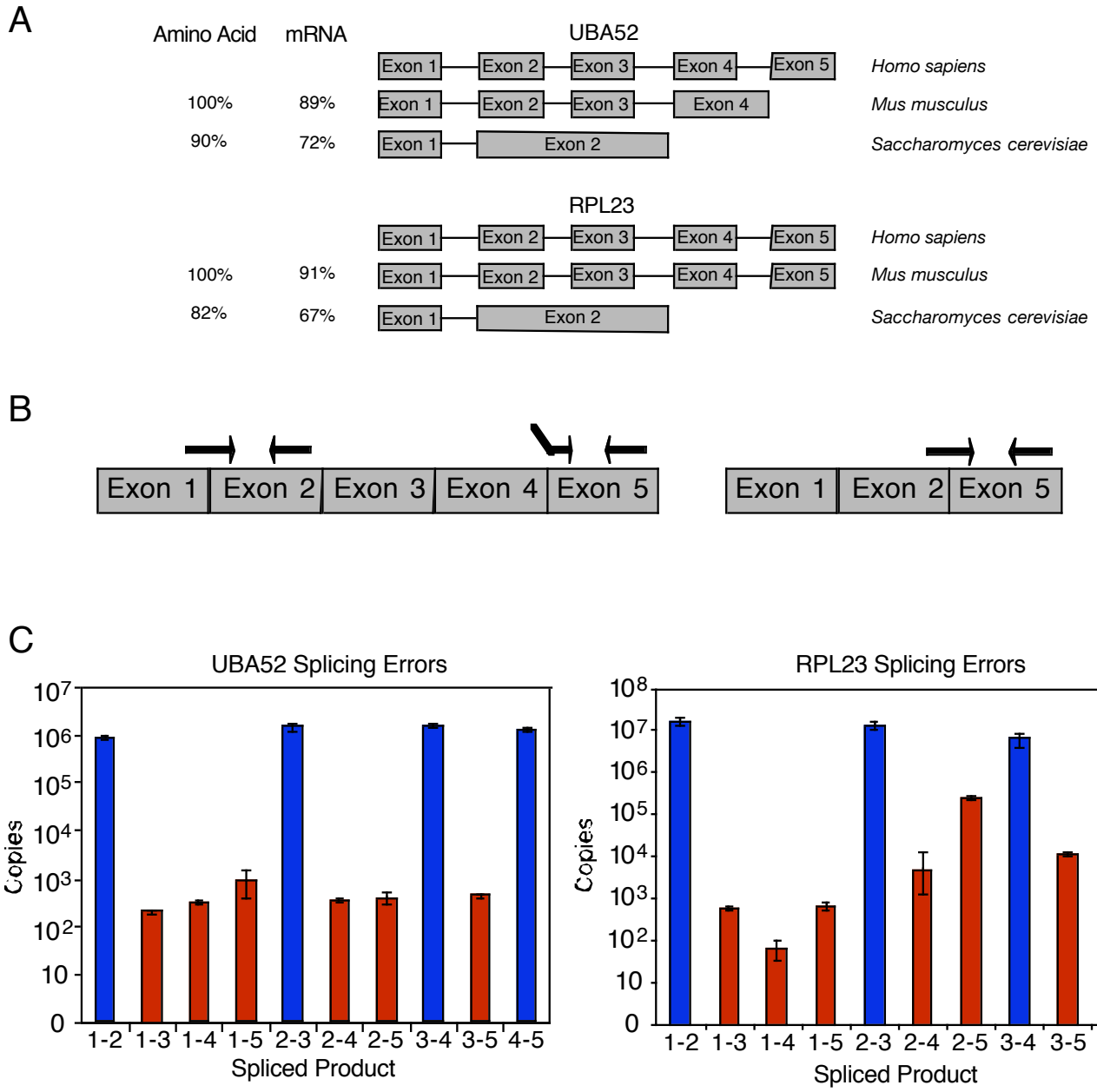

Figure 1. Error rates of pre-mRNA splicing. (A) The exon/intron structures and phylogenetic conservation of UBA52 and RPL23 are shown. (B) Experimental design using exon junction primers amplifying all mRNA isoforms generated through alternative exon ligation. Correct priming of the 1-2 splice variant is shown on the left. The middle set of primers shows incorrect priming of the 2-5 exon junction primer. The primers on the far right show correct priming of the 2-5 exon junction primer set. (C) Bar graph showing the total copy number detected for every exon skipping (red) and constitutive splicing (blue) event for all possible exon junctions in UBA52 and RPL23. cDNAs used to determine the error rate were generated with a mixture of random hexamers.

Task 3. Compare the splicing accuracy of normal breast cells and breast tumor cells.

After establishing a reliable and reproducible protocol to assess the accuracy of pre-mRNA splicing, we proceeded to compare the splicing fidelity of cell lines derived from breast cancer and matched control cell lines. Tumor (CRL-2336) and normal (CRL-2337) breast cell line pairs from ATCC® (the Global Bioresource Center) were grown side by side and equivalent concentrations of high quality cDNA were prepared. We then carried out real time PCR assays to determine the relative frequency of all possible *Uba52* and *RPL23* mRNA isoforms. The results demonstrate that breast cancer cell lines reproducibly demonstrate an up to 2.5-fold decrease in the number of errors made (Figure 2). One explanation for this observation could be that the observed change in splicing accuracy might be a consequence of altered transcription fidelity. This interpretation is currently tested. In sum, these results demonstrate that in breast cancer cell lines the fidelity of pre-mRNA splicing is modulated to permit less alternative splicing. That is, exons that are poorly recognized in normal cells will not be recognized in breast cancer, thus leading to altered pre-mRNA splicing patterns. The work thus far shows that alternative splicing changes observed in breast cancer can be explained by modulations in the accuracy of intron removal. These results suggest that breast cancer progression induces a general splicing perturbation that may increase the tumorigenesis. Based on this observations we will test the hypothesis that pre-mRNA splicing is influenced globally in breast cancer using microarray analysis.

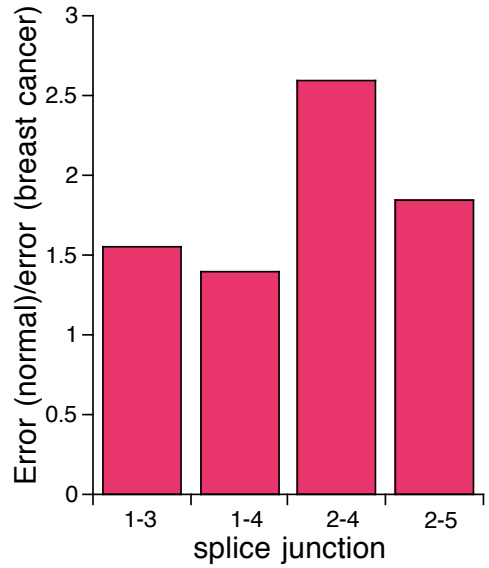

Figure 2. Splicing errors are decreased in breast cancer cells. The graph shows the relative change of splicing errors in breast cancer. All tested splice junctions display a reduced error rate.

Key Research Accomplishments:
-the accuracy of pre-mRNA splicing is limited by the fidelity of transcription by Pol II
-at its best the spliceosome makes a mistake once in 25,000 intron removal events
-the accuracy of intron removal is increased in breast cancer cells
-breast cancer is accompanied by a general pre-mRNA splicing defect that may contribute to tumorigenesis

Reportable Outcomes:
-portions of the work have been chosen for an oral presentation at this year's Cold Spring Harbor "Eukaryotic mRNA Processing" meeting, August 22-26, 2007 (abstract is attached)
-the work has been presented as part of a lecture given by the PI
-Kristi fox-Walsh, the student supported by the grant and the person carrying out the experiments defended her thesis in October 2007
-the exciting results from the work were the foundation for a RO1 application at NIH, funding decision pending

Conclusion:
The support of the proposed experiments has resulted in two exciting findings. First, we were able to determine the error frequency of the spliceosome, a fundamental piece of knowledge that has been elusive for more than 25 years of studying pre-mRNA splicing. Contrary to predictions in the field, the spliceosome is highly accurate in mediating intron removal, limited by the accuracy of nucleotide incorporation during pre-mRNA synthesis. The major conclusions from this set of experiments are that all multiple intron containing pre-mRNAs are alternatively spliced in human, albeit with drastically varying efficiencies, and that efficient alternative splicing is mediated through suboptimal splicing signals. The second exciting result is that the accuracy of pre-mRNA splicing in breast cancer is modulated, resulting in a general splicing defect. Future experiments using array approaches will identify the identity and significance of these splicing alterations.

References:
N/A

Appendices:
Copy of abstract of the work presented during the Cold Spring Harbor "Eukaryotic mRNA Processing" meeting, August 22-26, 2007

THE ACCURACY OF SPLICING IS LIMITED BY THE FIDELITY OF TRANSCRIPTION.

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Alternative pre-mRNA splicing generates thousands of different mRNA isoforms in metazoan organisms. The high proportion of pre-mRNAs that undergo alternative splicing necessitates an evaluation of the accuracy at which the spliceosome carries out intron removal. On one hand, it is possible that the spliceosome of higher eukaryotes is promiscuous at recognizing splice sites, thus allowing for the evolution of multiple alternative splicing patterns. On the other hand, the high levels of alternative splicing could be a consequence of splice sites that have evolved to offer a weak binding potential for components of the spliceosome, and the splicing machinery actually removes introns with a high degree of fidelity. We have designed a quantitative real-time PCR assay to determine the number of incorrectly spliced mRNA products made from pre-mRNA transcripts. To evaluate errors in splicing we examined pre-mRNA transcripts that 1) are >90% conserved throughout evolution, 2) produce only a single mRNA product in all eukaryotic genomes, 3) are ubiquitously expressed in all tissues, and 4) their alternative mRNA isoforms are not targeted by NMD. Analysis of all possible alternative exon exclusion patterns for these genes demonstrates that in some cases a splicing mistake is made only once in 15,000 intron removal events. These results demonstrate that the error rate of the spliceosome is extremely low and suggests that the accuracy of splicing is limited by the fidelity of transcription. Using this assay we examined splicing error rates in Spinal Muscular Atrophy (SMA). SMA is an autosomal recessive disease caused by the deletion of one of the two copies of the survival motor neuron (*SMN*) gene. In SMA patients, the remaining *SMN2* copy is unable to compensate for the loss of *SMN1*. Given the importance of SMN for snRNP assembly and regeneration, we hypothesized the loss of *SMN2* increases the number of miss-splicing events. Using SMA patient cells and RNAi treated cell lines we show that low concentrations of SMN result in an increase in the number of splicing errors. Thus, perturbed pre-mRNA splicing in SMA is mediated in part through alterations of the intrinsic fidelity of the spliceosome.